Microbial aspects of atrazine biodegradation in relation to history of soil treatment

Abdellah Yassir,[1†] Bernard Lagacherie,[1] Sabine Houot[2] and Guy Soulas[1]*

[1]*INRA-CMSE, Laboratoire de Microbiologie des sols, 17 rue Sully, BV 1540, 21034 Dijon Cedex, France*
[2]*INRA, Laboratoire de la Chaire de Science du sol, INA-PG centre de Grignon, 78850, Thiverval-Grignon, France*

Abstract: Among 15 soils with different cropping practices, seven which had an history of repeated atrazine applications showed accelerated degradation of this herbicide. By contrast, grassland or agricultural soils with no recorded atrazine application, at least for the last three years, had a low degradation potential. No direct relation was found between the rate of atrazine mineralisation and the size of the microbial biomass. In adapted soils, the amounts of extractable residues were lowered and the very high percentages of radioactivity from [*ring*-^{14}C]atrazine recovered as [^{14}C]carbon dioxide demonstrated that *N*-dealkylation and deamidation were the only processes for micro-organisms to derive carbon and energy for heterotrophic growth. Kinetics of microbial ^{14}C accumulation revealed that atrazine ring carbon could be incorporated by direct oxidative condensation with structural components of the bacterial or fungal cell whereas side-chain carbon was preferentially used for biosynthesis of new protoplasmic cell material, as confirmed by the high turnover rate of radiolabelled microbial components. From the determination of the Michaelis–Menten parameters, V_m and K_m in the presence of different selective biocides, it was possible to conclude that fungi were probably less active in atrazine degradation than bacteria and that over years the microbial atrazine-degrading community showed an increased efficiency.

Keywords: atrazine mineralisation; accelerated degradation; atrazine C biological fixation; extractable residues; enumeration (MPN); heterotrophic activity; K_m; V_m

1 INTRODUCTION

Atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) is a widely used herbicide to control a variety of broadleaf weeds infesting corn, sorghum and other crops. Its widespread use has caused environmental concern on the basis of frequent detection of atrazine in surface water,[1,2] rainwater,[3] tile drainage[4] and ground water[5,6] at concentrations frequently exceeding the EU permissible limit of water contamination (0.1 μg litre^{-1}) and the US-EPA maximum contaminant level (3 μg litre^{-1}) in force since 1992.[7]

Atrazine has been classified as a moderately persistent herbicide, with half-lifes ranging from several days to several months.[8–11] Recently, however, there have been reports of rapid atrazine mineralisation.[12,13] Enrichment culture techniques[14–16] have permitted the isolation of consortia[17,18] and pure bacterial isolates[19,20] capable of complete mineralisation of the chemical. Soil management, such as successive maize cropping and repeated annual atrazine applications[12,13] have been suspected of playing a role in the differential development of more efficient atrazine-degrading strains, whereas nitrogen fertilisation has been shown to regulate their activity (Abdelhafid *et al*, pers comm 1999)

It is now generally accepted that the alkyl side chains are the only sources of carbon and energy available to micro-organisms from atrazine through oxidation.[21] Atrazine ring nitrogen has also been reported to be used as a nitrogen source by soil microflora.[14,15] However, to date, there is no report on incorporation levels of ring or chain carbons within the soil biomass during mineralisation, especially for soils showing accelerated degradation of atrazine. Recently, enrichment with acclimated microbial species has been demonstrated by most probable number (MPN) enumeration techniques.[22] Yet, the main physiological characteristics of the emerging degrading microflora are not known, nor are their possible changes in response to increased selective pressure resulting from a long-term history of herbicide applications.

This paper reports on atrazine degradation in soils subjected to different cultivation practices. The aim of this investigation was to compare the kinetics of mineralisation of ^{14}C-ethyl side chain- and ^{14}C-ring-labelled atrazine in these soils and to relate them to the relative amounts of ^{14}C residues present as microbial

* Correspondence to: G Soulas, INRA-CMSE, Laboratoire de Microbiologie des sols, 17 Rue Sully, BV 1540, 21034 Dijon Cedex, France
†Present address: Université Caddi-Ayyad, Faculté des Sciences Semlalia, Département de Biologie, BPS 15, Marrakech, Maroc
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Table 1. History of crop succession and atrazine applications for the different soils investigated

Soil	*Crop*	*Atrazine applications*
Dijon		
Citeaux	Fallow	No atrazine for last 5 years
Arceau	Fallow	No atrazine for last 3 years
Grignon		
G4	Corn (no N fertilisation)	Atrazine since 1987
G10	Corn (N: 174 kg ha^{-1} $year^{-1}$)	Atrazine since 1987
G62	Corn	Atrazine since 1962
G65	Wheat	No atrazine since 1965
G73	Rotation: corn-wheat	Atrazine on corn since 1973
Saône valley		
VS (fallow)	Natural fallow field	No atrazine
VS2	Corn	Atrazine for last 2 years
VS3	Corn	Atrazine for last 3 years
VS7	Corn	Atrazine for last 7 years
Others		
Baccon	Rotation: corn-wheat	Atrazine on corn since 1988
Chigy	Corn	Atrazine since 1989
Bouzule 1 (silty)	Rotation: corn-wheat-barley	Alachlor and bentazone or metolachlor on corn but no atrazine
Bouzule 2 (clay)	Rotation: corn-wheat-barley	Alachlor and bentazone or metolachlor on corn but no atrazine

components or as extractable transformation products. We have also tried to determine whether the size of the degrading community, assessed by enumeration experiments, or its physiological characteristics, estimated through the determination of the Michaelis–Menten parameters (K_m and V_m) for atrazine mineralisation, control the appearance of accelerated degradation.

2 EXPERIMENTAL METHODS

2.1 Soils

Soil samples were collected in the 0–15 cm top layer of 15 cultivated and grassland soils from different areas in France. These differed in the recorded number and frequency of atrazine applications. The main soil properties, crop and herbicide histories are reported in Tables 1 and 2. Fresh soils were sieved, and only the fraction of aggregates between 2 and 3 mm was kept and stored at 4 °C. Unless otherwise stated, soil samples (20 g, oven-dry basis) were made of soil aggregates to maintain good aeration. The water content was adjusted to (100-x)% of the water holding capacity (WHC; measured by centrifugation at 1000 g, INRA-Laboratory for Soil Analysis, Arras, France), x being the part of the WHC which was covered by the volume of water needed for treating the soil with the chemicals.

2.2 Chemicals

Chemical grade non-labelled D-glucose (Rectapur®, Prolabo) and [U-^{14}C]D-glucose (spec act: 370 MBq $mmol^{-1}$, chemical purity >99%, Isotopchim) were used for heterotrophic activity measurements.

Soil	*Clay (%)*	*Silt (%)*	*Sand (%)*	*Organic C (g kg^{-1})*	*Total N (g kg^{-1})*	*pH (water)*
Citeaux	20.3	56.6	23.1	9.7	0.93	8.2
Arceau	31.1	64.8	4.1	12.6	1.39	7.2
G4	26.5	52.8	20.7	13.0	1.20	8.5
G10	27.8	56.2	16.0	11.9	1.32	8.3
G62	27.2	60.4	12.4	16.0	1.30	8.2
G65	27.6	52.2	20.2	24.2	1.84	8.1
G73	27.5	57.5	15.0	20.5	1.53	8.0
VS (fallow)	39.5	26.4	15.8	39.1	4.32	8.0
VS2	32.7	56.8	7.2	28.2	3.19	7.9
VS3	44.2	39.8	5.6	32.1	3.85	8.0
VS7	36.6	51.6	9.4	25.4	3.02	8.0
Baccon	37.1	57.1	5.7	15.6	1.7	7.2
Chigy	41.2	51.0	5.8	64.5	6.52	7.8
Bouzule 1	24.2	48.0	8.5	14.0	ND[a]	6.2
Bouzule 2	51.7	35.7	3.9	13.0	ND	7.3

Table 2. Main physical and chemical characteristics of the different soils

[a] ND: Not determined.

Analytical grade atrazine (chemical purity 99%, Pestanal®, Riedel de Haen) was used for preparing solutions of the herbicide after mixing with the appropriate ^{14}C radioactive chemical, either uniformly labelled on the ring (spec act: 659 MBq mmol^{-1}, purity 95%, Amersham-France) or on the ethyl side chain (spec act: 1776 MBq mmol^{-1}, purity 95%, Amersham-France).

2.3 Total microbial biomass measurements

For each soil, extractable microbial carbon and its extractability coefficient, K_{EC}, were determined in order to assess the size of the total soil biomass, using the Fumigation-Extraction procedure after preliminary in-situ labelling of the soil microflora, as described previously.[23] Briefly, the microbial biomass was first labelled by addition to each soil sample (20 g) of an aqueous solution (3 ml) of [^{14}C]glucose to obtain a final radioactivity content of 25 kBq kg^{-1} soil and a concentration (1 mg [^{14}C]glucose kg^{-1} soil) small enough to prevent any microbial growth. For each soil, six replicates were prepared in glass jars (250 ml). They were incubated for 24 h in biometer flasks (1 litre) which contained two polyethylene scintillation vials, one with water (10 ml) to maintain saturation, the other with a solution of sodium hydroxide (0.2 M; 10 ml) to trap [^{14}C]carbon dioxide evolved from mineralisation of [^{14}C]glucose. Incubation was performed in the dark in a climatic chamber at controlled temperature (20 (± 0.5) °C). After 24 h, which has been proved long enough to obtain maximum incorporation of C-glucose into the microbial biomass, the radioactivity content in the alkali solutions was determined by scintillation counting, and three of the samples were submitted to overnight (16 h) ethanol-free chloroform fumigation. All six fumigated and unfumigated samples were then extracted by shaking on an orbital shaker (30 min at 25 °C) with potassium sulfate solution (25 mM; 100 ml). The soil suspension was centrifuged (5000 rev min^{-1}) and the supernatant filtered through glass microfibre filters (Whatman). Aliquots (1 ml) were analysed for radioactivity content by scintillation counting. The dissolved organic carbon contents C(fumigated) and C(unfumigated) extracted from the fumigated and unfumigated samples were measured by persulfate oxydation under UV light. The K_{EC} coefficient and the microbial biomass, B, were calculated with the following formulae, assuming that total radioactivity initially added to the soil can only be converted into microbial carbon and [^{14}C]carbon dioxide:[23,24]

$$K_{EC} = \frac{(^{14}C(\text{fumigated}) - ^{14}C(\text{unfumigated}))}{(^{14}C(\text{initial}) - ^{14}CO_2 - ^{14}C(\text{unfumigated}))}$$

and:

$$B = \frac{(C(\text{fumigated}) - C(\text{unfumigated}))}{K_{EC}}$$

2.4 Determination of kinetics of atrazine mineralisation and incorporation of atrazine carbon into the microbial biomass

A series of identical soil samples were treated with labelled atrazine. The pesticide was added as a water solution to obtain an initial atrazine concentration of 0.5 mg kg^{-1} and a radioactivity content of 333.5 kBq kg^{-1}, the same for both types of radiolabelled compound. For each soil type, a number of samples were prepared and incubated under the same conditions as described above and the kinetics of [^{14}C]carbon dioxide formation were followed. Half-lives ($t_{1/2}$) for the mineralisation 'reactions' were calculated as described elsewhere[13] from the formula, $t_{1/2} = \text{Ln}2/k$, where k is the rate constant estimated using a first-order rate model for product formation. After different periods of incubation (0, 30 and 50 days in the Citeaux and Arceau soils, 8, 22, 36 and 50 days in the soils from Grignon and 7, 15, 25, and 40 days for all other soils) six replicate samples of the same soil were sacrificed, with three of them being fumigated. Percentages of radioactive carbon found in the microbial biomass were determined by subtracting percentages of extractable ^{14}C in unfumigated samples from percentages of extractable ^{14}C in fumigated samples and dividing by the K_{EC} coefficient, as determined with glucose. It was assumed that extractability of microbial carbon did not depend on the chemical nature of the carbon source.

2.5 Determination of the content in extractable residues

The amounts of water- and methanol-extractable residues were also determined. A series of soil samples (20 g of soil aggregates) received the appropriate volume of an aqueous solution of [*ring*-^{14}C]atrazine (0.5 mg kg^{-1} and 333.5 kBq kg^{-1}) and were incubated under the same conditions as previously described. Periodically, three replicate samples of the same soil were extracted in centrifuge glass tubes (250 ml) by overnight shaking in calcium chloride solution (40 ml, 0.01 M) on an orbital shaker (20 °C). After centrifugation (5000 rev min^{-1}; 10 min) the extracted radioactivity was analysed by scintillation counting. An equivalent volume of methanol was added to the centrifuge tube for another extraction (16 h, 20 °C). After centrifugation (5000 rev min^{-1}, 10 min) the amounts of methanol-extractable ^{14}C-residues were determined by scintillation counting.

2.6 Enumeration of atrazine degraders

Enumeration of micro-organisms capable of degrading atrazine was made by a most probable number (MPN) procedure using microtiter plates for sequential dilutions.[25] Preliminary experiments showed that the number of degraders in soils with a low capacity to degrade atrazine was very often below the detection limit of the procedure. For this reason enumeration was attempted only for soils in which accelerated mineralisation has been shown to occur. The presence

of degraders was detected from their ability to generate [^{14}C]carbon dioxide from ring-labelled atrazine.

Aliquots (0.1 ml) of a phosphate buffer solution (pH 8) were delivered into each of the 96 wells of sterile microtiter plates. A sample from each soil (40 g) was first diluted to half in sterile water. Aliquots (0.1 ml) of this soil suspension were added to each of the first series of eight wells. After mixing, 11 other serial two-fold dilutions were performed by transferring half the content of one series of eight wells to the next one for a maximum dilution of $1/2^{13}$ equivalent to 1.2×10^{-4}. Aliquots (0.1 ml) of an aqueous solution of pure [*ring*-^{14}C]atrazine were delivered to each well at the dose of 60 Bq per well (and a concentration less than 0.05 mg litre^{-1}). Microtiter plates were covered with a filter paper (Whatman) impregnated with a saturated solution of barium hydroxide (>56 g litre^{-1}) tightly maintained with a metallic lid fixed by clamps over the plate. After 30 days of incubation at 20 °C in a climatic chamber, the presence of radioactive spots on the filter papers was detected by autoradiography after exciting phosphor screens which were used for determining the radioactivity present on each spot using a phosphorimager (Storm 820®, Molecular Dynamics). Quantification of radioactivity was made using a calibration curve based on deposits of known amounts of radioactivity. The number of degraders was determined from the last positive dilutions by using statistical tables.[25]

2.7 Heterotrophic activity measurements and biokinetic parameters determination

We tried to determine the physiological characteristics of the atrazine-degrading community through the determination of the Michaelis–Menten parameters, V_m and K_m, considered as indicators of the capacity of the soil microflora to mineralise atrazine. Only soils showing accelerated degradation were compared and ring-labelled atrazine was used.

The parameters V_m and K_m were determined from the response curve of the initial rate of mineralisation of atrazine against its initial soil concentration, interpreted in terms of Michaelis–Menten rate law.[26,27] Samples (1 g of soil aggregates) from the adapted soils were placed in each well of 24-well microtiter plates. The water content (100% of WHC) was adjusted with the solution of atrazine. For each soil, eight different concentrations of atrazine were applied (0.5, 0.57, 0.75, 1, 1.35, 2, 3 and 5 mg kg^{-1}) with the same radioactivity (2 kBq) added to each well. The microtiter plates were covered by a filter paper (Whatman) initially soaked in a saturated barium hydroxide solution and tightly maintained over the wells by clamping a metallic lid to the plate. Soil incubations were made at 20 °C in climatic chambers. Mineralisation was measured after one week of incubation by measuring the radioactivity contained in the spots on the filter paper. Radioactivity content was determined with a phosphorimager as explained for enumeration experiments (Section 2.6). Rates of mineralisation, v, were estimated from the mean daily amounts of radioactivity evolved as [^{14}C]carbon dioxide. The experimental procedure was applied to assess the mineralising potential of the whole microbial community. In the same experiment we also tried to distinguish between the fungal and the bacterial contribution to the overall mineralising activity by treating the soil three days before atrazine addition either with a mixture of bactericides (polymixin B: 0.1 g kg^{-1}, chloramphenicol: 2 g kg^{-1}, oxytetracycline: 2.5 g kg^{-1}, penicillin G: 1.25 g kg^{-1}) or with a fungicide (cycloheximide: 2.5 g kg^{-1}).[28] The Michaelis–Menten rate law was chosen to express the relation between initial rates of mineralisation, v, versus initial atrazine concentration, C:

$$v = V_m C/(K_m + C)$$

Estimates of the biokinetic parameters V_m and K_m with their statistical significance were obtained using the statistical module of SigmaPlot 4 (Jandel Scientific) for non-linear regression analysis.

3 RESULTS AND DISCUSSION

3.1 Basic microbial characterisations

The value of the K_{EC} coefficient, reflecting carbon extractability from microbial cells, was calculated after in-situ labelling of the soil microflora with [^{14}C]glucose. It varied between 0.37 and 0.49, with a mean value of 0.44 and a coefficient of variation of less than 10% (Table 3). This was in agreement with the values which have been found for different topsoils, varying from 0.20 to 0.50.[29,30] The different soils could be discriminated on the basis of their microbial carbon content which varied from 167 mg kg^{-1}, in an experimental plot in Grignon which had received neither inorganic nor organic fertilisation since 1875, to 1282 mg kg^{-1} in a grassland rhizospheric soil in the Saône valley (Table 3).

3.2 Kinetics of atrazine mineralisation and formation of residues in relation to previous agricultural practices

Kinetics of ^{14}C mineralisation from ethyl- or ring-labelled atrazine are reported in Fig 1 and corresponding half-lives in Table 3. Eight of the 15 soils that have been tested showed accelerated degradation. Half-lives for mineralisation of ethyl- and ring-labelled material are between 5.7 and 20.7 days on the one hand and 4.6 and 12.2 days on the other. All these soils had a history of atrazine treatment at least for the last two years, some of them receiving annual applications of this herbicide for 36 years. In these soils, mineralisation of [*ring*-^{14}C]atrazine reached 65–85% of the initial radioactivity. These results corroborate observations of enhanced biodegradation after several previous field applications or even after only one prior amendment with atrazine.[12,13] During the same period, 60–70% of the initial radioactivity present as

Table 3. K_{EC} values, total biomass, half-lives for atrazine mineralisation and MPN counts of [*ring*-^{14}C]atrazine degraders of the different soils[a]

			$t_{1/2}$ (days) (± SD)		
Soil	K_{EC} (± SD)	Biomass (mgC kg^{-1} soil)	ethyl-^{14}C	ring-^{14}C	MPN (cells g^{-1} soil)
Citeaux	0.360 (±0.005)	250	37.8 (±2.8)	ID	ND
Arceau	0.400 (±0.006)	247	ID	ID	ND
G4	0.425 (±0.005)	167	6.6 (±0.8)	5.5 (±0.8)	37300
G10	0.441 (±0.007)	207	6.8 (±1.2)	5.7 (±1.1)	11200
G62	0.493 (±0.004)	193	7.6 (±2.4)	6.7 (±2.5)	1240
G65	0.469 (±0.009)	272	ID	ID	ND
G73	0.471 (±0.009)	243	5.7 (±0.8)	4.6 (±0.8)	480
VS (fallow)	0.443 (±0.004)	1282	53.3 (±4.5)	ID	ND
VS2	0.488 (±0.002)	196	9.5 (±2.1)	5.9 (±1.4)	670
VS3	0.444 (±0.003)	608	20.7 (±8.3)	12.2 (±4.3)	ND
VS4	0.473 (±0.006)	658	14.1 (±3.9)	9.3 (±2.5)	3640
Baccon	0.411 (±0.007)	433	7.5 (±1.1)	5.5 (±0.9)	7120
Chigy	0.382 (±0.003)	528	24.5 (±6.3)	ID	ND
Bouzule 1	0.438 (±0.005)	213	112 (±27)	ID	ND
Bouzule 2	0.390 (±0.009)	449	49 (±4.2)	ID	ND

[a] ND: Not determined ID: Determination not possible.

^{14}C-ethyl side chain was mineralised. By contrast, other soils had a comparatively low degradation potential. Half-lives for mineralisation of side-chain-labelled atrazine vary between 24.5 days and 112 days when calculations are possible. For these soils, mineralisation data were not adequately fitted using the first-order rate model and half-lives for ring-labelled atrazine mineralisation were impossible to calculate. One of these soils, the Chigy soil, which has a very high organic carbon content, had received repeated atrazine applications since 1989. The low mineralisation rate could probably reflect a limited bioavailability of atrazine for microbial metabolism as a result of strong interactions with soil organic matter. An inverse relation between soil organic matter content and atrazine mineralisation has been demonstrated after compost addition which results in the stabilisation of atrazine residues mainly through sorption processes.[31] Other low-degrading soils were either grassland soils or agricultural soils from fields cultivated with continuous wheat crop or with wheat–maize crop rotation with no recorded atrazine application, at least for the last three years. In these 'non-adapted' soils, aliphatic ethyl side chain C was always mineralised faster and to a greater extent than aromatic C. Moreover, no direct link could be detected between the rates of mineralisation and any of the measured soil properties (Table 1) including the size of the total microbial biomass (Table 3).

For soils showing enhanced degradation, the same microbial population or community is likely to perform dechlorination, dealkylation and cleavage of the ring, with no limiting step in the metabolic pathway giving identical patterns for the kinetics of ^{14}C mineralisation from both the side-chain and the ring. Two different metabolic routes have been found for complete mineralisation of triazines by microbial species which are able to use it as a carbon and nitrogen source. After a first dealkylation, *Rhodococcus corallinus* is able to dechlorinate deethylsimazine and deisopropylatrazine with an hydrolase, TrzA, and to complete degradation by deamination, dealkylation and cleavage of the cyanuric acid heterocycle.[32,33] *Pseudomonas* sp ADP proceeds through dechlorination with the chlorohydrolase AtzA and hydrolysis of the side-chains with other amidohydrolases, AtzB and AtzC, producing cyanuric acid and finally carbon dioxide and ammonia.[14,15,34,35] The finding of very high percentages of mineralisation obtained with [*ring*-^{14}C]atrazine as compared to [*side-chain*-^{14}C]atrazine corroborates the fact that only compounds with alkylamino groups or hydrogen atoms bonded to the C atoms in the ring have non-zero reductance degree. *N*-dealkylation and deamidation are the only processes for micro-organisms to derive the carbon, nitrogen and energy for heterotrophic growth.[21] By contrast, for soils with no adapted micro-organisms, the higher percentages of mineralisation obtained with [*ethyl*-^{14}C]atrazine and the longer lag observed with [*ring*-^{14}C]atrazine suggested that dealkylation was the first step of transformation, and that ring cleavage was limiting.[36]

Changes with time in the amounts of aqueous- and methanol-extractable radioactivity are reported in Fig 2. In adapted soils, radioactivity recovery of both the aqueous and methanol-extractable ^{14}C-residues did not exceed 5% at the end of the experiments, with only 10–15% of the initial radioactivity remaining as non-extractable residues, as already reported.[12] Comparatively, in soils with no evidence of accelerated degradation, the amounts of extractable residues which have been found were between 25 and 40% of

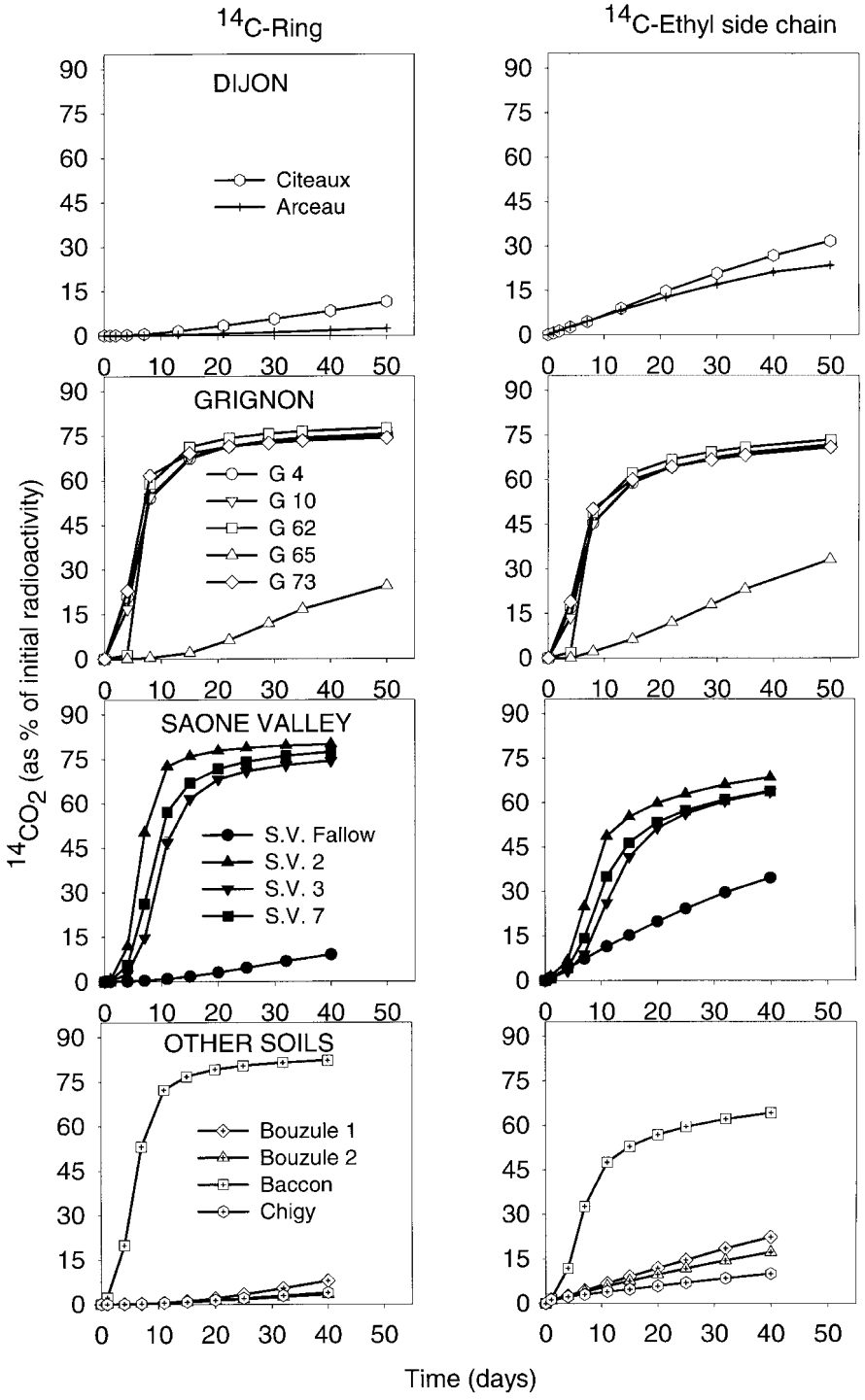

Figure 1. Kinetics of mineralisation of ^{14}C-ethyl- and of ^{14}C-ring-labelled atrazine in different agricultural soils (Standard deviations are plotted as vertical bars when larger than the symbols).

the initial radioactivity in both the aqueous and methanol extracts. In these soils, the final percentage of radioactivity as non-extractable residues represented between 15 and 50%, with a mean value of 30%. Again, the Chigy soil showed a peculiar behaviour with a very low amount of water-extractable residues, which reflects the low availability of atrazine and its metabolites in this soil. Between 40 and 50% of these residues remained extractable with methanol. These results do not corroborate those found elsewhere for the same soils and presenting a more detailed description of the distribution of the different metabolites (Houot *et al*, pers comm 1999).

3.3 Comparison of incorporation kinetics for ring- and side-chain atrazine carbon

Kinetics of accumulation of radioactive microbial carbon (Fig 3) supported previous conclusions. In soils showing accelerated mineralisation of atrazine, the use of ring-labelled chemical results in a constant proportion at a level not exceeding 5% of the initial radioactivity being found in microbial cells throughout the experiment. When using chain-labelled atrazine, 20–25% of the initial radioactivity was present in the biomass one week after atrazine application. A rapid decrease of the microbial radioactive carbon content followed, to reach a final level of 5–10% of the initial

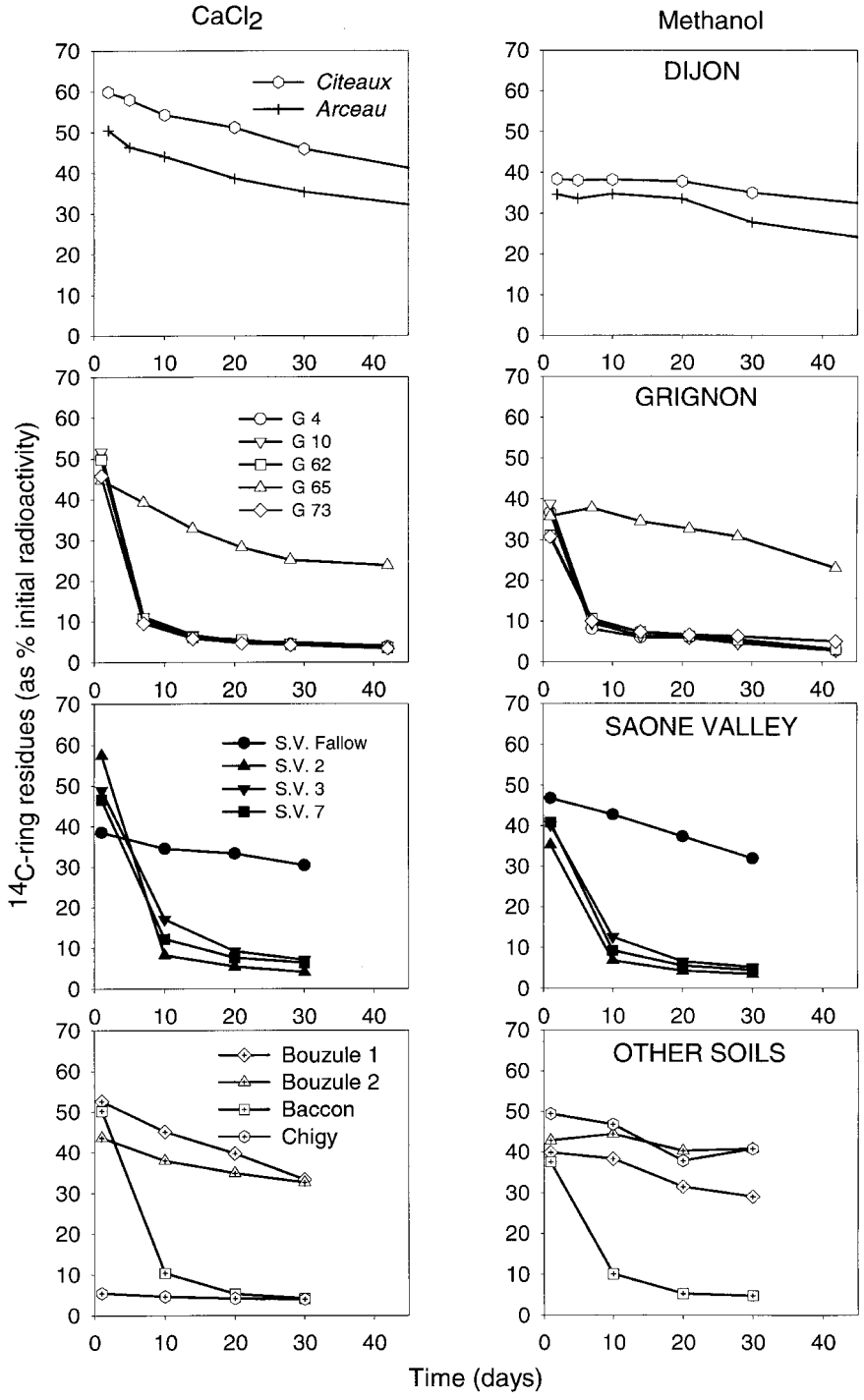

Figure 2. Temporal changes in the percentage recovery of total radioactivity found as aqueous-and methanol-extractable ^{14}C-ring-labelled atrazine residues (Standard deviations are plotted as vertical bars when larger than the symbols).

radioactivity. A different pattern was obtained when dealing with soils where adapted micro-organisms were not present or inactive. With both types of labelled atrazine there was a net increase in the proportion of the initial radioactivity which could be identified as microbial carbon as determined by the fumigation–extraction procedure. Non-adapted soils could be distinguished by their kinetics of radioactivity incorporation: soils from the Dijon area and from Grignon showed an initial lag phase (one to three weeks) followed by an increase in microbial ^{14}C content which was still perceptible after 50 days of incubation. Soils from all other experimental sites showed microbial ^{14}C incorporation starting with no apparent lag and levelling off after two to three weeks to reach final levels varying between 15 and 20% of the initial radioactivity. The Chigy clay organic soil (Fig 2f) is an exception, with a maximum incorporation of less than 5% for side-chain carbon, whereas erratic values were obtained for ring carbon (not shown).

These results gave evidence that, in adapted soils, atrazine could be used as carbon source by micro-organisms and that carbon from both the side-chain and the ring was incorporated into cellular components. Different mechanisms are likely to be involved. First of all, it is possible that residual parent atrazine or

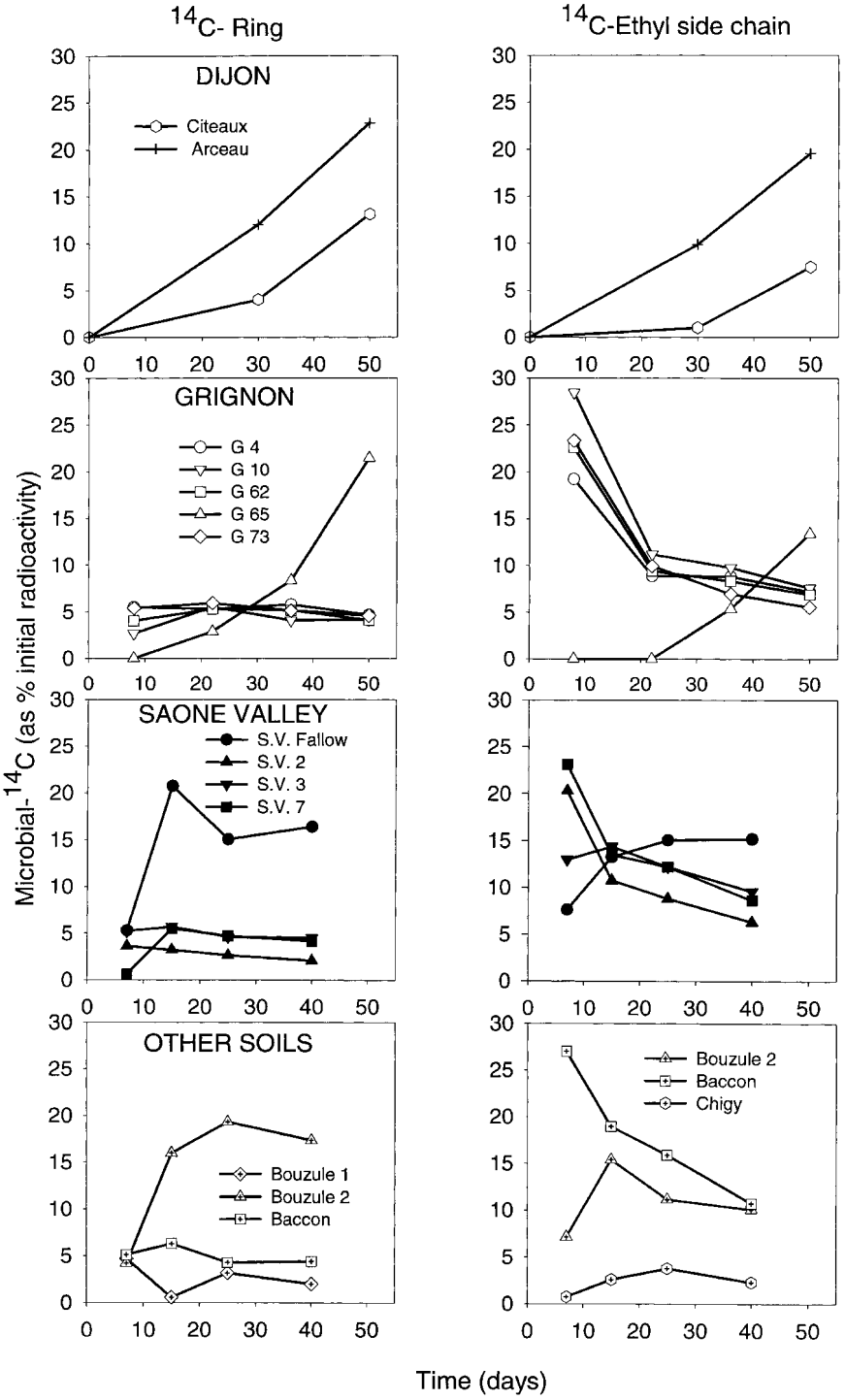

Figure 3. Kinetics of ^{14}C-incorporation into the soil biomass after application of ^{14}C-ethyl- and ^{14}C-ring-labelled atrazine in different agricultural soils (Standard deviations are plotted as vertical bars when larger than the symbols).

immediate metabolites present as cytoplasmic solutes liberated by fumigation could account for part of the radioactivity extracted by the FE technique, which does not discriminate in the chemical nature of the extracted organic material. Yet, the four- to five-fold increase in the percentages of radioactivity found in the microbial biomass one week after treatment with ethyl- as compared to ring-labelled atrazine strongly suggested that other predominant processes for biological incorporation of side-chain carbon were involved. From the use of ring-labelled atrazine a maximum of about 5% of the initial radioactivity as parent compound or aromatic derivatives could be expected. Yet, other processes may contribute to ring carbon fixation into cellular components. If assimilation by biosynthetic routes is to be ruled out due to the reductance degree of atrazine ring carbon,[21] direct oxidative condensation of atrazine or of its amino or hydroxy derivatives with humic-like components of the bacterial or fungal cell is possible. The constant proportion of radioactivity found in the microbial compartment is in agreement with a localisation on microbial compounds with a low turnover rate. This process has been demonstrated in pure culture and in

Table 4. Values of the Michaelis–Menten parameters, V_m and K_m, for the kinetics of [*ring*-^{14}C]atrazine mineralisation

	Total microflora		*Bacteria*		*Fungi*	
Soil	V_m (mg Al day^{-1}kg^{-1} soil)	K_m (mg Al kg^{-1} soil)	V_m (mg Al day^{-1}kg^{-1} soil)	K_m (mg Al kg^{-1} soil)	V_m (mg Al day^{-1}kg^{-1} soil)	K_m (mg Al kg^{-1} soil)
G4	0.56 (±0.04)	4.56 (±0.55)	0.28 (±0.02)	2.21 (±0.30)	0.012 (±0.001)	0.46 (±0.09)
G10	0.91 (±0.11)	8.00 (±1.33)	0.58 (±0.04)	4.75 (±0.56)	0.011 (±0.001)	0.24 (±0.08)
G62	0.66 (±0.05)	7.72 (±0.81)	0.07 (±0.005)	0.79 (±0.17)	0.0006 (±0.0001)	0.04 (±0.08)
G73	0.24 (±0.01)	3.54 (±0.36)	0.01 (±0.001)	0.04 (±0.08)	ID[a]	ID
VS2	0.08 (±0.01)	0.26 (±0.11)	0.03 (±0.001)	0.39 (±0.10)	ID	ID
VS7	0.14 (±0.01)	4.10 (±0.47)	0.02 (±0.001)	0.09 (±0.07)	ID	ID

[a] ID: Determination not possible.

soil for 2,4-D metabolites,[37–39] and has also been confirmed for atrazine in an aquatic environment.[40] On the other hand, there is no thermodynamic limit for micro-organisms to use for biosynthesis side-chain carbon which may contribute to the creation of new protoplasmic material during the period of active degradation of atrazine. After disappearance of the herbicide, the high turnover rate of labelled microbial components could explain the decrease of this pool of radioactivity. In non-adapted soils, the observed parallelism between kinetics of radioactivity incorporation from ring and side-chain carbon is not in opposition with the assumption of the coexistence of two different modes for the biological incorporation of radioactivity. It can be postulated that *N*-dealkylation preceding side-chain carbon assimilation is a limiting step controlling liberation and binding of amino derivatives of atrazine on microbial material.

3.4 Enumeration and heterotrophic activity of atrazine degraders. Determination of biokinetic parameters for atrazine mineralisation

Soils showing accelerated degradation had very small atrazine-degrading communities varying from 480 cells g^{-1} in the G73 soil from the Grignon area to 37,300 cells g^{-1} in the G4 soil from the same area (Table 3). These figures obtained for microbial communities having ring-cleavage activity were similar to those reported in previous studies.[41] As demonstrated in those studies no relationship appeared between total microbial biomass and the size of the atrazine-degrading community, which was evidently not influenced by the number of previous annual atrazine applications, since 1987 in G4 and G10 soils, since 1962 in G62 soil and since 1973 in G73 soil. The higher number of degraders found in the G4 soil which had not received nitrogen fertilisation since 1875, could signify that nitrogen limitation in soil increased the selective advantage for micro-organisms that can use atrazine as a nitrogen source (Abdelhafid *et al* pers comm 1999). Yet, the size of the degrading community was not correlated with the final percentages of mineralisation and incorporation, which were very close for all adapted soils. The number of atrazine-degrading micro-organisms did not limit the mineralisation process. The soils from the Saône valley seemed to behave in a different way, with a smaller number of degraders associated with significantly lower biomass and percentage of incorporation. Yet these differences were not reflected in significant differences in kinetics of mineralisation, which were again very similar.

The parameters V_m and K_m, respectively the maximum rate of mineralisation when atrazine concentration is not limiting and the Michaelis–Menten constant reflecting the affinity of the enzyme system for the chemical, are good physiological indicators of the atrazine-degrading community. Estimated values of these parameters together with their standard errors calculated for the total microflora and for the bacterial and fungal communities of the 'adapted soils' from the Grignon area and the Saône Valley are reported in Table 4. From these data it can be concluded that fungi contributed less to atrazine mineralisation than did bacteria. Evolution of radioactivity from soils treated with a bactericide was detected at a significant level for only two of the six investigated soils. This was reflected in V_m values for the fungal microflora which either could not be determined or were at least one or two orders of magnitude less than those found for the bacterial and the whole microflora. However, the contribution of the bacterial microflora could not account for the total mineralising activity, suggesting a possible synergy between fungi and bacteria. Because deleterious side effects of the fungicide mixture on bacteria were also possible, we did not try to go further in the discussion of the respective degrading activity of the bacterial and fungal communities. Only parameters which were determined in the absence of biocides can be regarded with the necessary confidence to be compared and discussed. For the total microflora, the overall *mass heterotrophic quotient* for atrazine mineralisation (the ratio 10^3 V_m/B) varied from 6.0 for the G4 soil to 4.4 for the G10 soil, 3.4 for the G62 soil and 1 for the G73 soil. It showed a decreasing level of specificity of the total microflora with the number of previous atrazine applications. This observation contrasted with the higher degree of specialisation of the degrading microflora reflected in the overall *enumerative heterotrophic quotient* for atrazine mineralisation (the ratio 10^5 V_m/MPN) which was 1.5 in the G4 soil, 8.1 in the G10 soil but increased to 53.2

and 50 in the G62 and G73 soils. VS2 and VS7 soils which had been treated for less than 10 years maintained a degrading microflora which exhibited heterotrophic quotients of the same order of magnitude (12 for VS2 and 3.8 for VS7) as those found in G4 and G10 soils. This observation supported the conclusion that, over the years, degradation became more and more rapid as the result of a selection process which allowed emergence of more efficient degrading species which either pre-existed at a very low level in the soil or resulted from naturally occurring gene transfer for which indirect[42] and direct[43] evidence has already been obtained for another xenobiotic compound, 2,4-D. Interestingly, alternate wheat cropping, as occurred in G73 soil, did not seem to modify appreciably the potential for adaptation of the soil microflora. Furthermore, K_m values which were related to the affinity of the degrading species towards the herbicide did not change as adaptation proceeded, indicating that, at the overall community level, *r*-strategists with improved growth rate are likely to be selected.

4 CONCLUSION

Atrazine has been recognised as a major contaminant of surface and ground waters. The main cause is to be found in its relatively long persistence in soils with half-lives extending over weeks or even months. Our investigations confirmed that accelerated degradation of this herbicide has appeared rather frequently in agricultural soils where atrazine has been repeatedly used for weed control. As far as its biological activity is not affected, this offers new perspectives for safer use of this herbicide based on rapid and complete mineralisation without significant accumulation of undesirable metabolites.

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